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In Vitro and Intracellular Antioxidant Activities of Brown Alga *Eisenia bicyclis*

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Abstract

The antioxidant activities of a methanolic extract of *Eisenia bicyclis* and its organic solvent fractions, including dichloromethane (CH_2Cl_2), ethyl acetate (EtOAc), *n*-butanol (*n*-BuOH), and water (H_2O) fractions, were investigated. Scavenging activities against DPPH, hydroxyl, superoxide anion, and peroxynitrite radicals were evaluated using electron spin resonance spectrometry; intracellular reactive oxygen species (ROS) were evaluated by a 2′,7′-dichlorofluorescein diacetate assay using RAW264.7 mouse macrophages. The antioxidant activities of the individual fractions were: EtOAc>*n*-BuOH>CH_2Cl_2>H_2O. The EtOAc fraction exhibited strong radical scavenging activity and a significantly reduced ROS level in RAW264.7 cells. Moreover, the phenolic contents of the extract and fractions followed the same order as their radical scavenging activities. Our results indicate that *E. bicyclis* is a valuable natural source of antioxidants that may be applicable to the functional food industry.

Key words: Antioxidant activity, *Eisenia bicyclis*, Electron spin resonance, RAW264.7

Introduction

Free radicals are produced by endogenous factors, such as normal respiration, and exogenous factors, such as the metabolism of foreign materials, smoking, and ultraviolet (UV) radiation (Pryor, 1986; Robinson et al., 1997). Inspired molecular oxygen reacts readily with free radicals to generate reactive oxygen species (ROS), including superoxide anion radical, hydroxyl radical, singlet oxygen, and hydrogen peroxide (H_2O_2), as well as reactive nitrogen species (RNS), including peroxynitrite, formed by the reaction of nitric oxide and superoxide anion in the body (Sawa et al., 2000; Yildirim et al., 2000). These ROS and RNS can cause oxidative damage to several cellular components, including lipids, proteins, and nucleic acids, and induce inflammation or lesions in various organs (Beckman et al., 1990). These reactive species are also associated with various degenerative diseases, including cancer, aging, arteriosclerosis, rheumatoid arthritis, and allergy (Dreher and Junod, 1996; Griffiths and Lunec, 1996; Squadrito and Pryor, 1998; Sohal, 2002).

Recently, there has been great interest in marine resources as a source of powerful and nontoxic natural antioxidants, given their reported inhibition of oxidative stress and aging-induced disorders. Numerous crude extracts and pure compounds obtained from marine resources have been reported to possess antioxidant and radical scavenging activities (Lim et al., 2002; Kang et al., 2004; Kuda et al., 2005; Duan et al., 2006; Ganesan et al., 2008; Zou et al., 2008). In particular, marine algae are of great interest due to their diverse natural products, unique structure, and biological abilities based on their antioxidant activity.

Eisenia bicyclis (Kjellman) Setchell is a perennial brown

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alga belonging to the family Laminariaceae. This species is widely distributed throughout Korea and Japan, but it is particularly abundant on Ulleung Island in South Korea. *Eisenia bicyclis* is frequently used as a foodstuff, along with *Ecklonia cava* and *Ecklonia stolonifera*. In previous studies, *E. bicyclis* was shown to have various bioactive derivatives, including phlorotannins, polysaccharides, pyropheophytin, peptides, and oxylipin. In addition, many beneficial bioactivities have been attributed to *E. bicyclis*, including the inhibition of inflammation, hyaluronidase and diabetic complications (Whitaker and Carlson, 1975; Noda et al., 1989; Kojima et al., 1993; Shibata et al., 2002; Kousaka et al., 2003; Okada et al., 2004). Among these derivatives, phlorotannins, which are tannins composed of polymerized phloroglucinol units, have been shown to possess strong antioxidant activity, possibly due to their unique structures (Ahn et al., 2007). However, limited information is available concerning their antioxidant and radical scavenging activities. The aim of the present study was to investigate the antioxidant activities of a methanolic (MeOH) extract of *E. bicyclis* and its solvent soluble fractions using various *in vitro* and intracellular free radical scavenging activity assays. In addition, the total phenolic contents of the extract and fractions were determined to clarify the relationship between their antioxidant activities and phlorotannin contents.

Materials and Methods

Materials

Eisenia bicyclis was purchased from Ullengdomall (Ulleng Island, Korea) in March 2008. The sample was ground to a particle size of <50 μm and the powder was stored in a freezer at -20°C until use. DPPH, 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO), $FeSO_4$, and 2′,7′-dichlorofluorescein diacetate (DCFH-DA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The mouse macrophage cell line RAW264.7 was obtained from the American Type Culture Collection (Manassas, VA, USA). All materials required for cell culture were obtained from Gibco BRL, Life Technologies (Rockville, MD, USA). Dihydrorhodamine 123 (DHR 123) was purchased from Molecular Probes (Eugene, OR, USA). Authentic peroxynitrite was obtained from Cayman Chemicals (Ann Arbor, MI, USA). All other reagents were of the highest grade available commercially.

Preparation of the MeOH extract and fractions

The lyophilized powder of *E. bicyclis* (3.8 kg) was extracted three times with 10 L of hot MeOH. The MeOH extract (624.3 g) was then partitioned with organic solvents to yield dichloromethane (CH_2Cl_2, 170.5 g), ethyl acetate (EtOAc, 90.4 g), and *n*-butanol (*n*-BuOH, 100.8 g) fractions, in addition to an H_2O layer (262.6 g).

DPPH radical scavenging activity assay

DPPH radical scavenging activity was measured using the method of Nanjo et al. (1996). A 30 μL sample (or ethanol itself as a control) was added to 30 μL of DPPH (60 μM) in ethanol. After mixing vigorously for 10 s, the solution was transferred to a 100 μL quartz capillary tube and the scavenging activity of the samples toward DPPH radicals was measured using a JESFA electron spin resonance (ESR) spectrometer (JEOL, Tokyo, Japan). The spin adduct was measured on an ESR spectrometer exactly 2 min later. The experimental conditions were: magnetic field, 336.5±5 mT; power, 5 mW; modulation frequency, 9.41 GHz; amplitude, 1×1,000; and sweep time, 30 s. The DPPH radical scavenging ability of each sample was calculated according to the following equation:

$$\text{DPPH scavenging activity (\%)} = (1-A/A0)\times 100,$$

where A and $A0$ were the relative peak heights of radical signals with and without sample, respectively. The percent scavenging activity was plotted against the sample concentration to obtain the IC_{50}.

Hydroxyl radical scavenging activity assay

Hydroxyl radicals were generated by the iron-catalyzed Fenton Haber-Weiss reaction, and the radicals were rapidly reacted with a nitrone spin trap using DMPO. The resultant DMPO-OH adducts were detected with an ESR spectrometer (Rosen and Rauckman, 1984). The sample solution (20 μL) was mixed with DMPO (0.3 M, 20 μL), $FeSO_4$ (10 mM, 20 μL), and H_2O_2 (10 mM, 20 μL) in a phosphate buffer solution (pH 7.4) and then transferred to a 100 μL quartz capillary tube. After 2.5 min, the ESR spectrum was recorded using an ESR spectrometer. The experimental conditions were: magnetic field, 336.5±5 mT; power, 1 mW; modulation frequency, 9.41 GHz; amplitude, 1×200; and sweep time, 4 min. The scavenging activity was calculated as follows:

$$\text{Hydroxyl radical scavenging activity (\%)} = (1-A/A0)\times 100,$$

where A and $A0$ were the relative peak heights of radical signals with and without sample, respectively.

Superoxide radical scavenging activity assay

Superoxide radicals were generated using a UV-irradiated riboflavin/EDTA system (Guo et al., 1999). The reaction mixture containing 0.3 mM riboflavin, 1.6 mM EDTA, 800 mM DMPO and the indicated sample concentration was irradiated for 1 min under a UV lamp at 365 nm. The reaction mixture was then transferred to a 100 μL quartz capillary tube for measurement by ESR spectrometry. The experimental conditions were: magnetic field, 336.5±5 mT; power, 10 mW; modulation

frequency, 9.41 GHz; amplitude, 1×1,000; and sweep time, 1 min. The superoxide radical scavenging ability of each sample was calculated as follows:

Superoxide radical scavenging activity (%)=(1-*A*/*A*0)×100,

where *A* and *A*0 were the relative peak heights of radical signals with and without sample, respectively.

Peroxynitrite scavenging activity assay

Peroxynitrite scavenging activity was assessed according to a modified version of Kooy's method (Kooy et al., 1994). Highly fluorescent rhodamine 123, which is rapidly oxidized from non-fluorescent DHR 123 in the presence of peroxynitrite, was monitored. In brief, the rhodamine buffer (pH 7.4) used in this assay consisted of 50 mM sodium phosphate dibasic, 50 mM sodium phosphate monobasic, 90 mM sodium chloride, 5 mM potassium chloride, and 100 μM diethylenetriaminepentaacetic acid. The final DHR 123 concentration was 5 μM. The buffer used in this assay was freshly prepared and maintained on ice. The samples were dissolved in 10% dimethyl sulfoxide (DMSO) at 12.5-100 μg/mL for the extract/fractions. The fluorescence intensity of the oxidized DHR 123 was evaluated using a GENios microplate reader (Tecan Austria GmbH, Grödig, Austria) at excitation and emission wavelengths of 480 and 530 nm, respectively. All values are expressed as the mean±SD of three experiments.

Quantification of the total phenolic content

The total phenolic contents were determined via a modified version of the Folin-Ciocalteu method using gallic acid as a standard (Singleton and Rossi, 1965). A 0.1 mL aliquot of the extract was mixed with 1 mL of Folin-Ciocalteu reagent (previously diluted 1:1 [v/v] with water and 2 mL of 20% sodium carbonate [Na_2CO_3] solution). The mixed solution was maintained at room temperature for 45 min, followed by 10 min of centrifugation at 5,000 rpm. The absorbance of the supernatant was measured at 730 nm using a GENios microplate reader (Tecan Austria GmbH). The total phenolic contents of the fractions are expressed as a percentage (wt %) compared to the weight of the dried extract or fraction.

Cell culture

RAW264.7 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% heated-inactivated fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 μg/mL) at 37°C in a humidified atmosphere of 95% air and 5% CO_2. The medium was changed every other day.

Assessment of cell cytotoxicity

Cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, which is based on the conversion of MTT to formazan crystals by mitochondrial dehydrogenases (Mosmann, 1983). Cells were cultured in 96 well plates (1.5×10^5 cells/well) with serum free media and treated with different concentrations of sample for 24 h. The MeOH extract of *E. bicyclis* and its solvent fractions were dissolved in 10% DMSO. The final concentration of DMSO in the culture medium never exceeded 0.1%. Sequentially, 100 μL of MTT dye solution was added to each well. After 4 h of incubation, 100 μL of DMSO was added to dissolve the formazan crystals and the absorbance was read using a GENios microplate reader (Tecan Austria GmbH) at 540 nm.

Measurement of the intracellular ROS-scavenging activity using DCFH-DA labeling

To assess intracellular ROS formation, the redox sensitive fluorescent probe DCFH-DA was used as described by LeBel et al. (1992). RAW264.7 cells were incubated with 50 μM DCFH-DA for 30 min at 37°C in the dark. Following three washes with PBS, the cells were incubated with different samples (100 μg/mL) for 1 h then mixed with 500 μM H_2O_2. The level of intracellular ROS in the collected cells was determined using a FACSCalibur™ flow cytometer (488 nm excitation, 530 nm emission) equipped with CELLQUEST analysis software (Becton Dickinson, Mountain View, CA, USA). For each treatment, 10,000 cells were counted. The results are expressed as the average H_2DCF-DA fluorescence intensity in the cells to determine the free radical scavenging activities of the samples.

Results and Discussion

In the present study, we focused on the antioxidant effects of a MeOH extract of *E. bicyclis* and its organic solvent fractions. The antioxidant effects of the solutions were assayed for ROS, including DPPH, hydroxyl, superoxide anion, and peroxynitrite radicals, using ESR spectrometry. Furthermore, a DCFH-DA assay was carried out to assess their intracellular ROS scavenging effects using a flow cytometer and RAW264.7 mouse macrophages.

Free radicals and their major ROS are unstable and react with various macromolecules in the human body (Halliwell and Gutteridge, 1990). In fact, free radicals and ROS or RNS, including hydroxyl radical, superoxide anion and peroxynitrite, play a crucial role in the etiology of several diseases (Beckman et al., 1990), including cancer, Alzheimer's disease, rheumatoid arthritis, and atherosclerosis (Squadrito and Pryor, 1998). Thus, the scavenging of free radicals and ROS is probably one of the most effective defenses in the body against disease. ESR spectroscopy is used to evaluate chemical spe-

cies such as free radicals or inorganic complexes possessing a transition metal ion. In recent years, ESR has been widely used to measure free radical levels in radical scavenging activity assays due to the convenience and high sensitivity of the technique (Sachindra et al., 2007).

DPPH radical scavenging activity

DPPH is a stable free radical that has been extensively used to assess the antioxidative activities of antioxidants (Antolovich et al., 2002). As summarized in Table 1, the scavenging activity of the MeOH extract (ex.) and its solvent soluble fractions (fr.) toward DPPH radicals was as follows: EtOAc fr.>*n*-BuOH fr.>CH_2Cl_2 fr.>MeOH ex.>H_2O fr., with IC_{50} values of 27.0±0.9, 29.8±0.3, 44.1±0.6, 239.6±0.6, and >500 μg/mL, respectively. These results indicate that the EtOAc fraction of the MeOH extract possessed the strongest scavenging activity toward DPPH radicals. The *n*-BuOH fraction also exhibited noticeable scavenging activity; however, the H_2O fraction showed no activity.

Hydroxyl radical scavenging activity

Hydroxyl radicals are the shortest lived and most highly reactive ROS; they react rapidly with biological molecules and can attack cellular molecules, including hepatic tissue (Hippeli and Elstner, 1997). Hydroxyl radicals were generated by the Fenton reaction ($Fe^{2+}+H_2O_2 \rightarrow Fe^{3+}+OH^-+\cdot OH$) and trapped by a stable radical, DMPO, forming nitroxide adducts that were detected by ESR spectrometry (Makino et al., 1991). As shown in Table 1, the extract and fractions exhibited potent or moderate scavenging activity against hydroxyl radicals. The order of the IC_{50} values for the extract and fractions was: EtOAc fr. (22.0±0.3 μg/mL)>*n*-BuOH fr. (24.8±0.3 μg/mL)>MeOH ex. (29.5±0.2 μg/mL)>CH_2Cl_2 fr. (36.6±0.1 μg/mL)>H_2O fr. (101.5±0.8 μg/mL). Among the fractions, the EtOAc and *n*-BuOH fractions were most potent in their inhibitory effects against hydroxyl radicals. In contrast, the H_2O fraction exhibited weak scavenging activity.

Superoxide anion scavenging activity

Superoxide anion is a precursor of single oxygen and hydroxyl radicals and a weak oxidant that indirectly initiates lipid peroxidation. The presence of superoxide anion can magnify cellular damage because it increases other free radicals and oxidizing agents (Zou et al., 2008). The superoxide anion scavenging activity of the extract and fractions are shown in Table 1. The potential scavenging activity of the extract and fractions were: EtOAc fr.>*n*-BuOH fr.>MeOH ex.>CH_2Cl_2 fr.>H_2O fr., with IC_{50} values of 14.6±2.5, 246.6±4.6, 301.1±3.5, 302.7±1.6, and 325.8±3.9 μg/mL, respectively. The EtOAc fraction showed the highest level of activity; the H_2O fraction showed the lowest.

Peroxynitrite scavenging activity

Peroxynitrite, formed from the reaction of superoxide with nitric oxide, is cytotoxic (Squadrito and Pryor, 1998). The need for a strong peroxynitrite scavenger is clear due to the absence of an enzyme that can exert protective effects against damage induced by peroxynitrite. As shown in Table 1, the IC_{50} values were found to be 17.2±0.6, 36.9±0.5, 5.4±0.8, 9.4±0.7, and 72.4±1.2 μg/mL for the MeOH ex., CH_2Cl_2 fr., EtOAc fr., *n*-BuOH fr., and H_2O fr., respectively. The order of peroxynitrite scavenging activity was EtOAc fr.>*n*-BuOH fr.>MeOH ex.>CH_2Cl_2 fr.>H_2O fr. Among the fractions, the EtOAc and *n*-BuOH fractions exhibited potent scavenging activities against peroxynitrite; in contrast, the H_2O fraction exhibited moderate scavenging activity.

The total phenolic contents

Phenolic compounds are common in plants and have been reported to have various bioactivities, including antioxidant activities. According to previous studies, marine algae and their polyphenols have antioxidant properties (Yan et al., 1999; Lim et al., 2002; Kuda et al., 2005). The major active polyphenols in algae extract are phlorotannins (Yan et al., 1999). The total phenolic contents of the MeOH extract of *E. bicyclis* and its solvent soluble fractions are shown in Table 2. The greatest total phenolic content was identified in the EtOAc fr. (68.8±0.3%), followed by the *n*-BuOH fr. (35.3±1.8%), MeOH ex.

Table 1. Radical scavenging effects of the MeOH extract (ex.) of *Eisenia bicyclis* and its solvent fractions (fr.)

Samples	IC_{50} (μg/mL±SD)			
	DPPH	Hydroxyl	Superoxide	$ONOO^-$
MeOH ex.	239.6 ± 0.2	29.5 ± 0.2	301.1 ± 3.5	17.2 ± 0.6
CH_2Cl_2 fr.	44.1 ± 0.6	36.6 ± 0.1	302.7 ± 1.6	36.9 ± 0.5
EtOAc fr.	27.0 ± 0.9	22.0 ± 0.3	14.6 ± 2.5	5.4 ± 0.8
n-BuOH fr.	29.8 ± 0.3	24.8 ± 0.3	246.6 ± 4.6	9.4 ± 0.7
H_2O fr.	> 500	101.5 ± 0.8	325.8 ± 3.9	72.4 ± 1.2

Table 2. Total phenolic contents of the MeOH extract (ex.) of *Eisenia bicyclis* and its solvent fractions (fr.)

Samples	Total phenol contents (%)*
MeOH ex.	34.2 ± 0.6
CH_2Cl_2 fr.	13.4 ± 1.0
EtOAc fr.	68.8 ± 0.3
n-BuOH fr.	35.3 ± 1.8
H_2O fr.	7.8 ± 0.7

*Data were expressed as percentage (wt %) compared to weight of dried extract or fractions.

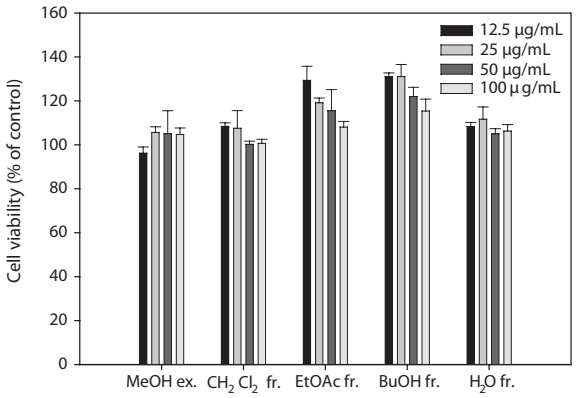

Fig. 1. Effects of the MeOH extract (ex.) of *Eisenia bicyclis* and its solvent fractions (fr.) on cell viability in Raw 246.7 cells. Values are expressed as the mean±SD of triplicate experiments.

(34.2±0.6%), CH_2Cl_2 fr. (13.4±1.0%), and H_2O fr. (7.8±0.7%). Notably, the order of the total phenolic contents is similar to that of antioxidant activities. Several studies have shown a close relationship between antioxidant activity and the total phenolic content (Negro et al., 2003; Toor and Savage, 2005).

Cytotoxicity of the extract and fractions toward RAW264.7 cells

The cytotoxicities of the tested extract and fractions were assessed using RAW264.7 cells to evaluate the endocellular action of antioxidation. Our results show that the tested extract and fractions had no cytotoxic effects, even at a concentration of 100 μg/mL. In addition, a marked difference could not be found between the tested extract and fractions and the control (Fig. 1).

Cellular ROS determination using DCFH-DA

To evaluate the direct radical scavenging effects of the MeOH extract and its solvent fractions in cells, DCFH-DA was used as the substrate to measure intracellular ROS production in neutrophils. DCFH-DA freely penetrates into cells and is hydrolyzed to dichlorofluorescin (DCFH) by intracellular esterases. This non-fluorescent dye is then oxidized to fluorescent dichlorofluorescin (DCF) by the action of cellular ROS. RAW264.7 cells were selected to investigate the effects of phlorotannins in the MeOH extract on the intracellular production of ROS. As shown in Fig. 2, the average DCF

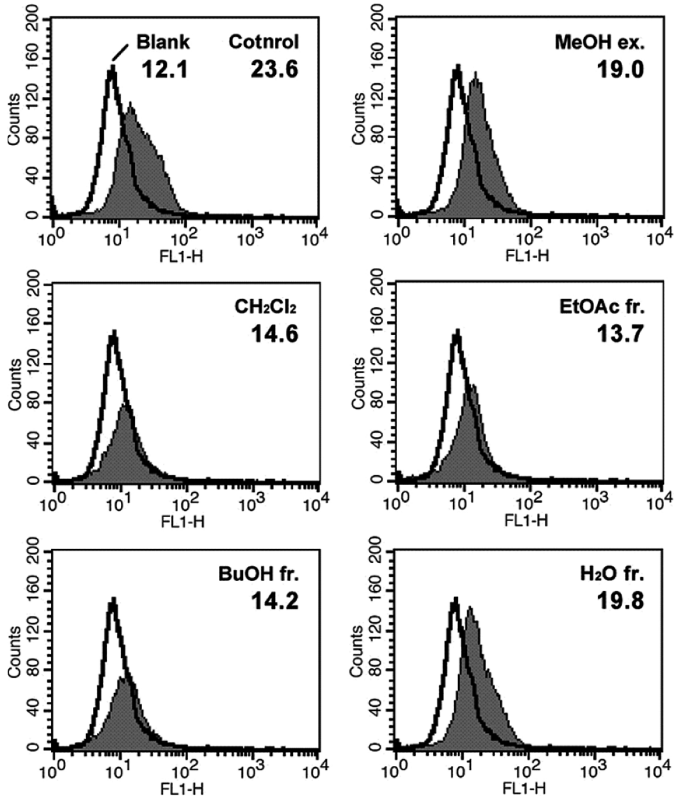

Fig. 2. Intracellular radical scavenging activities of the MeOH extract of *Eisenia bicyclis* and its solvent fractions in RAW 264.7 cells. Cells were labeled with oxidation sensitive dye, 2′,7′-dichlorofluorescein diacetate and treated each sample for 1 h. After washing the cells with PBS for three times, 500 μM H_2O_2 was added to cells (blank: -H_2O_2, control: +H_2O_2). The levels of intracellular reactive oxygen species in the collected cells were determined using flow cytometer (488 nm excitation, 530 nm emission). The results were expressed as the average H_2DCF-Da fluorescence intensities in the cells to determine the free radical scavenging activities of the samples.

fluorescence in the control, which was treated with 500 μM H_2O_2, was twice (23.6) that in the blank (H_2O_2 non-treated) after 2 h. However, pretreatment with the MeOH extract and its solvent fractions decreased the average DCF fluorescence, as compared to the control. This result indicates that the MeOH extract and its solvent fractions had considerable intracellular radical scavenging activities in RAW264.7 cells. As with our other radical scavenging assay, the MeOH extract and H_2O fraction showed moderate radical scavenging activities while the EtOAc fraction exhibited the highest radical scavenging activity (13.7). This result suggests that the radical scavenging activity of the EtOAc fraction, which had the highest total phenolic content (68.8±0.3%) (Table 2), was caused by phlorotannins. Together, our results suggest that the EtOAc fraction is a potent antioxidant material that can protect against the radical-mediated oxidation of cellular biomolecules and that contains various antioxidative phenolic compounds, including phlorotannins.

The present study demonstrated that a MeOH extract of *E. bicyclis* and its solvent soluble fractions exhibited antioxidant activity. Among the extract and fractions, the order of antioxidant activity in five radical scavenging assays was: EtOAc fr.>*n*-BuOH fr.>MeOH ex.>CH_2Cl_2 fr.>H_2O fr. This order is similar to that for the total phenolic contents of the extract and fractions. Phenolic compounds are common in edible and inedible plants and algae. The antioxidant activity of phenolics is mainly due to their redox properties, which include important roles as hydrogen donators, in the adsorption and neutralization of free radicals, and as oxygen (singlet and triplet) quenchers. They have been reported to have multiple biological effects, including antioxidant, anti cancer, cardio protective, and anti-inflammatory effects (Rice-Evans et al., 1997; Kähkönen et al., 1999; Yoon and Baek, 2005). These results suggest that *E. bicyclis* has strong potential as a functional food, dietary supplement, or medicine for the prevention or treatment of various diseases. However, the compounds responsible for the antioxidant activities of the extract and its fractions are unclear. Therefore, further study is needed to isolate and identify the active components of *E. bicyclis* for its application to the functional food industry.

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